Virulence of *Galleria mellonella* Nuclear Polyhedrosis Virus to Diamondback Moth, *Plutella xylostella* (L.) After Serial Passage in *Spodoptera frugiperda* Cells Cultivated *in vitro*

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ABSTRACT

In this study, *Galleria mellonella* nuclear polyhedrosis virus (*Gm*NPV) was serially passaged 20 times in *Spodoptera frugiperda* cell line (Sf9). At each passage of virus production, some of the replication characteristics were analyzed including the percentage of infection, the production of polyhedra and extracellular virus (ECV) titers. Also the comparative bioassay was done to determine the virulence of the various isolates of *Gm*NPV for the 2nd stage larvae of *Plutella xylostella*.

After 10 passages in cell culture, significant reductions in infection percentage and the polyhedra production were observed. In the 9th and 10th passages, the ECV production was increased significantly. The electron microscopic observation also showed the abnormality of polyhedra morphology at high frequency after 10 serial passages. The comparative bioassay revealed that the virulence of wild isolate (LC_{50} = 1.94x10^4 OBs/cm^2) was highest when compared to the plaque-purified isolate (LC_{50} = 4.71 x10^4 OBs/cm^2), the 1st passage isolate (LC_{50} = 5.68 x10^4 OBs/cm^2), the 5th passage isolate (LC_{50} = 6.75 x10^4 OBs/cm^2), and the 10th passage isolate (LC_{50} = 6.89 x10^4 OBs/cm^2). The LT_{50} showed that all isolates killed larvae at similar rates (P>0.05) with the shortest incubation time in the wild isolate (4.8 days) followed by the 1st passage isolate (5.1 days), the 10th passage isolate (5.1 days), the plaque-purified isolate (5.4 days) and the 10th isolate (5.6 days), respectively.

KEYWORDS: diamondback moth, *Plutella xylostella*, baculovirus, nuclear polyhedrosis virus, *Gm*NPV, insect cell line

1. INTRODUCTION

Diamondback moth, *Plutella xylostella* (L.), belongs to the family Yponomeutidae, order Lepidoptera. The insect is a cosmopolitan species that probably originated in the Mediterranean region. It is found over much of North America, the southern portion of South America, Southern Africa, Europe, Southeast Asia, New Zealand, and parts of Australia. Host plants of this pest include both cultivated and wild plants of the family Cruciferae, as well as several ornamentals, such as wallflower, candytuft, stocks, and alyssum. Cultivated crops that are attacked by this insect include broccoli, Brussels sprouts, cabbage, caulifiower, Chinese broccoli, Chinese cabbage, flowering white cabbage, head cabbage, mustard cabbage, and watercress. Weed hosts, such as

mustard and radish, are important reservoir hosts for the species [1]. Newly hatched caterpillars feed in the spongy plant tissue beneath the leaf surface forming shallow mines that appear as numerous white marks. The larvae are surface feeders in all subsequent stages. These larvae feed on the lower leaf surface 62-78% of time, chewing irregular patches in leaves[1]. All the leaf tissues are consumed except the veins. On some leaves, the larvae feed on all but the upper epidermis creating a "window" effect. The last stage larva is a voracious feeder, it causes more injury than the first three larval instars. Life stages of the diamondback moth vary considerably depending on the environment in which it develops. This insect pest prefers a warm environment for its development. The serious infestation usually occurs during dry season around February to April, when optimum climatic conditions prevail, especially little to no rain, and food plants are readily available [2].

The baculoviruses, especially nuclear polyhedrosis virus, have been repeatedly reported as effective and safe pest control agents [3-4]. These viruses possess characteristics which are suitable for use in integrated pest management programs. They are exceedingly specific and often highly virulent to their hosts. Among the baculoviruses, *Galleria mellonella* nuclear polyhedrosis virus (*Gm*NPV), normally infects the larvae of wax moth, but is also capable of causing disease in other insect hosts such as *Agrotis ipsilon*, *Heliothis virescens*, *Heliothis zea*, *Plathypena scabra*, *Pseudoplusia includens*, *Spodoptera litura*, *Spodoptera ornithogalli*, *Trichoplusia ni* and *Plutella xylostella* [5]. In Malaysia, *Gm*NPV was used in laboratory tests against *Plutella xylostella* larvae. Although pathogenicity of *Gm*NPV to diamondback moth is only marginal virulent, this virus shows faster speed of kill when compared with other viruses being used in the test [6]. The nuclear polyhedrosis viruses were up to recently mass-produced in insects. However, in order to be commercially viable, virus based insecticides must be produced on a large scale, conveniently and efficiently, under strictly controlled and reproducible conditions. The use of in vitro systems for virus production is currently recognized as being more suitable for large scale production of viral insecticides [7-9]. *Gm*NPV is easily produced using cell culture technology. Moreover, preliminary experiments conducted on *Gm*NPV produced in cell cultures adapted to serum-free medium demonstrated the virulence of this virus to *Plutella xylostella* species present in Taiwan [10]. The aim of this study was first to assess the virulence of *Gm*NPV produced in larvae and cell culture systems for *Plutella xylostella* from Thailand and moreover to assess the yield of production and the virulence of this baculovirus following serial viral passages *in vitro*.

2. MATERIALS AND METHODS

Insect cells

Sf9 cell line (clone of *Spodoptera frugiperda* IPLB-Sf21 cell line) was routinely maintained in Grace's medium (GIBCO) supplemented with 10% FBS, 0.0666 g/l lactalbumin hydrolysate, 0.0666 g/l yeastolate and 50 µg/ml of gentamycin. For subculturing the cells, the old medium was discarded from the flask and replaced with 5 ml of pre-warmed fresh medium. Sf9 cells usually grew loosely attached, and were easily resuspended by gentle pipetting and transferred to a new flask at a concentration of of 2×10^5 cells/ml.

Serial viral passage and light microscopic studies

Galleria mellonella nuclear polyhedrosis virus was used in the experiment. Five ml portions of Sf9 cell suspension were seeded in triplicate in 25 cm^2 plastic tissue culture flasks. Cells were incubated at 28°C overnight to allow their attachment to the flask surface. On the following day, the old tissue culture medium from the cell monolayer was replaced with 1 ml of virus inoculum. In the 1^{st} passage, virus inoculum was derived from the diluted hemolymph of *Galleria mellonella* larva infected with *Gm*NPV and in subsequent passages virus inoculum was obtained from infectious medium from previous passage. As control, Grace's medium was used instead of virus inoculum.

The cells were routinely examined with an inverted phase-contrast microscope and light micrographs of the infected cells were taken every 24 hours post infection.

Evaluation of viral infection

At 7 days post-infection, the cells were detached from the flasks by gently pipetting with medium for enumeration of infected and uninfected cells using hemacytometer and trypan blue exclusion method. The percentage of infection was then calculated. The remaining cell suspension from each flask was collected for the purification of occlusion bodies, according to a modified protocol described by Miller and Dawes [11]. Triplicate counts of occlusion bodies were performed using hemacytometer. Their concentration was adjusted to 10^7 OBs/ml in sterile distilled water, and kept as a stock solution at -20 °C.

In this study, the extracellular virus titers of *Gm*NPV were determined by the tissue culture infectious dose ($TCID_{50}$) as follows: The cell concentration was adjusted to 1×10^5 cells/ml with complete tissue culture medium. Each well of a 96-well microtitre plate (Corning™) was seeded with 100 µl aliquots of the cell suspension and incubated overnight at 28 °C. The following day, 10 µl aliquots of each virus dilution were added to each well of the plate. Ten serial dilutions were prepared from infected culture media of the different passages and 8 wells were used for each dilution of virus. Four replications were done for each virus dilution and plates were incubated at 28 °C for 7 days. Wells were then observed for the presence of polyhedra and the $TCID_{50}$ values were calculated by Karber method [12]. All the data obtained from serial passage study were submitted to statistical analysis by using ANOVA model and LSD test (with the significance level of 0.05) to determine the significant differences in each data series.

Electron microscopic analysis

Cells were harvested by centrifugation. Cell pellets were fixed with 2.5% glutaraldehyde in cacodylate buffer pH 7.2-7.4 at 4 °C for 1 hour, washed with cacodylate buffer pH 7.2-7.4 at 4°C for 10 minutes two times, post-fixed with 1.3% osmium tetroxide at room temperature for 1 hour and washed with distilled water. During each step of procedure cells were suspended in the solution after pelleting by centrifugation (at 7000 g for 5 minutes). Thereafter the distilled water were removed from the cells, they were dehydrated in a graded series of acetone solutions (25%-50%-75%-95%-100%-100%) and embedded in resin, and cut with diamond knife. The ultra thin sections were stained with uranyl acetate and lead citrate. They were examined and photographed with an electron microscope (Philips™ 300). The thin sections were observed for the polyhedra morphology and the relative amount of occluded virions.

Plaque purification of virus isolates

Four ml aliquots of Sf9 cell suspension were placed in 60 mm tissue culture dishes (Corning™) at a concentration of 2×10^6 cells/dish. A monolayer culture was induced by incubation at 28°C for 30 minutes. Ten serial dilutions of virus derived from 1st, 10th and 15th passage were prepared. The tissue culture medium from the plate was aspirated and 0.5 ml of virus inoculum was added. Each dilution was inoculated in duplicate. As control, 0.5 ml of tissue culture medium was added instead of virus. The cells were incubated at room temperature for 1 hour. The virus inoculum was then removed and 4 ml of 0.5% agarose overlay warmed to 40-42°C was added to each plate. The cells were then incubated at 28°C. After 5 days post-infection, plaques would appear as clear, circular areas. The well-isolated plaques on a plate that has 10 plaques or less were selected and examined under the microscope. The virus present in the plaques was aspirated using dropper and kept at –20°C.

Bioassay studies

Plutella xylostella larvae were collected from Klong Rangsit vegetable cultivated area, Pathumthanee province, Thailand. The larvae used throughout the experiments were maintained in the laboratory on Chinese kale leaves.

The virulence of *Gm*NPV to the second stage larvae of *Plutella xylostella* was examined by using leaf disk contamination technique [13]. Concentrations of occlusion bodies (OBs) of *Gm*NPV (10^7, 10^6 and 10^5 OBs/ml) derived from various virus isolates including wild type (from infected larvae of *Galleria mellonella*), 1st passage, 5th passage, 10th passage and plaque purified isolate were adjusted with sterile distilled water. A volume of 5 µl of each test concentration was applied to the surface of Chinese kale leaf disk (6 mm in diameter). After liquid evaporation, one second-instar larva was placed on each treated leaf disk. As control, 5 µl of sterile distilled water was applied on the leaf disk instead of virus. Each bioassay was replicated three times (twenty-five larvae per replication). After 24 hours, larvae that had consumed the entire exposed leaf area were transferred individually into another container containing fresh leaves and fed until death or pupation. Mortality was scored daily. To confirm diagnosis of *Gm*NPV infection, the dead larvae were smeared, and stained by using Buffalo black method [14]. The presence of infected OBs was microscopically observed at 100x under oil immersion. The Lethal Concentration 50 (LC_{50}) and Lethal Time 50 (LT_{50}) were estimated by the Probit analysis [15]. Chi-square tests were used to assess the goodness of fit of the model to the data. Significant differences in potency between different virus preparations was determined by one-way ANOVA and LSD test with significance level of 0.05 (Spss® 10.0.1 for Win98NT, 2000).

3. RESULTS

Galleria mellonella nuclear polyhedrosis virus was serially passaged through *Spodoptera frugiperda* cell line (Sf9) up to 20 times. As shown in Table 1, the percentage of infection decreased from 71.5 % to as low as 8 %. From the 1st passage through the 10th passage, the viral infection decreased slightly but declined sharply and significantly after approximately 10 viral passages.

At each passage of *Gm*NPV production, the infected cells were examined routinely by phase-contrast light microscope. The hypertrophy of nuclei and the presence of polyhedra were the criteria of the virus infection. The healthy uninfected Sf9 cells (untreated control) are illustrated in Figure 1a and infected cells from the 1st, 5th, 10th, 15th, and 20th passages at 48 hours post infection in respectively Figures 1b and 2a,b,c,d. In this study, the infection of *Gm*NPV was generally observed within 24 hours post exposure and the infected cells appeared to be filled with mature polyhedra from 48 hours post infection and mostly disrupted in the late time post infection.

In the early passages (1st passage up to 10th passage) a large number of polyhedra were produced in infected nuclei and there was just a small difference in the level of polyhedra production. The polyhedra formed in the infected cells in those passages were quite similar in size and shape.

In contrast, drastic reduction in polyhedra production in the infected nuclei was generally observed after the 10th passage (Figure 2). Some of the infected cells contained no polyhedra but exhibited typical signs of NPV infection such as hypertrophy of nuclei. Instead of forming polyhedra, many granular bodies appeared inside the cells. Moreover, the abnormal size (arrow) of polyhedra can be seen easily in cells infected by NPV passaged more than 10 times (Figure 2d).

The electron microscopic appearance of the *Gm*NPV infected cells obtained from the 1st, 5th, 10th, 15th, and 20th passages are shown in Figures 3 and 4. During the 1st passage, normal replication and polyhedra production were observed (Figure 3). Inside each polyhedron, many occluded viruses were presented within polyhedra and the polyhedra appeared in normal shape and size. From the 5th and 10th passages the viral replication and polyhedra production were quite similar to the 1st one (Figures 4a, 4b). After approximately 10 passages of virus *in vitro*, it became apparent that the viral replication and polyhedra produced were abnormal. As shown in Figures 4c and 4d,

Table 1 Serial passage of *Gm*NPV in Sf9 cells.

Virus passage No.	Percentage of infection	Total No. of polyhedra per culture (5 ml)	$TCID_{50}$/ml
1	69.8 ^{a}	$5.75x10^{6}$ ^{e}	$1.58x10^{8}$ ^{i}
2	71.5 ^{a}	$2.82x10^{6}$ ^{ef}	$4.68x10^{9}$ ^{ij}
3	64.9 ^{a}	$1.20x10^{6}$ ^{g}	$3.16x10^{7}$ ^{i}
4	49.1 ^{ab}	$1.20x10^{6}$ ^{g}	$3.16x10^{7}$ ^{i}
5	62.4 ^{ab}	$1.41x10^{6}$ ^{f}	$1.78x10^{8}$ ^{i}
6	58.3 ^{ab}	$1.15x10^{6}$ ^{g}	$1.58x10^{7}$ ^{i}
7	57.8 ^{ab}	$1.20x10^{6}$ ^{g}	$1.26x10^{8}$ ^{ij}
8	40.8 ^{b}	$3.16x10^{6}$ ^{e}	$1.58x10^{9}$ ^{j}
9	42.2 ^{b}	$1.20x10^{6}$ ^{g}	$1.58x10^{11}$ ^{j}
10	47.7 ^{b}	$1.29x10^{6}$ ^{g}	$1.58x10^{14}$ ^{l}
11	21.3 ^{d}	$1.20x10^{4}$ ^{h}	$1.99x10^{7}$ ^{i}
12	11.3 ^{d}	$1.29x10^{5}$ ^{h}	$5.01x10^{6}$ ^{i}
13	8.5 ^{d}	$1.29x10^{5}$ ^{h}	$2.51x10^{6}$ ^{i}
14	9.3 ^{d}	$6.03x10^{4}$ ^{h}	$1.99x10^{6}$ ^{k}
15	13.0 ^{d}	$6.03x10^{4}$ ^{h}	$1.99x10^{6}$ ^{k}
16	9.0 ^{d}	$8.91x10^{4}$ ^{h}	$1.48x10^{6}$ ^{k}
17	10.9 ^{d}	$2.57x10^{4}$ ^{h}	$3.98x10^{6}$ ^{k}
18	8.0 ^{d}	$3.98x10^{4}$ ^{h}	$1.58x10^{5}$ ^{k}
19	11.3 ^{d}	$7.94x10^{4}$ ^{h}	$6.31x10^{6}$ ^{i}
20	9.6 ^{d}	$2.51x10^{4}$ ^{h}	$1.58x10^{6}$ ^{k}

Mean values of % infection and polyhedra production were calculated from 3 replications, and mean values of ECV titer production was calculated from 4 replications. Means followed by the same letter were not significantly different (P>0.05).

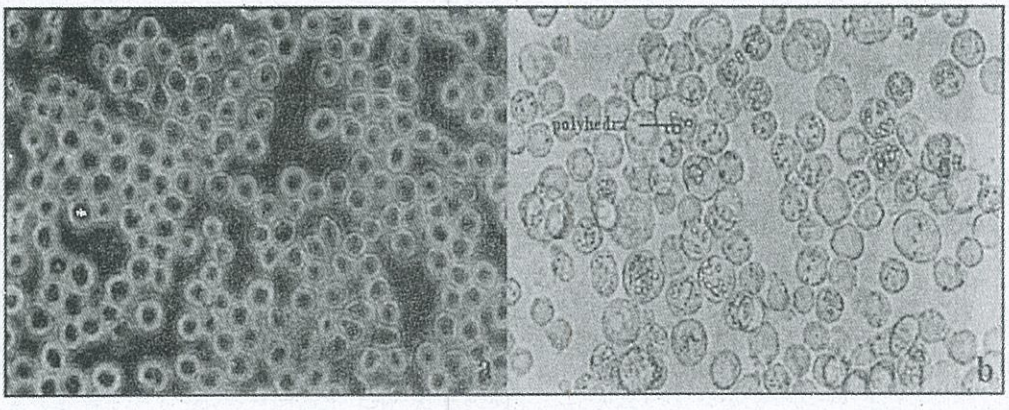

Figure 1 Phase-contrast micrographs of non-infected Sf9 cells (a), and at 48 hours post-*Gm*NPV (1st passage) infection (b)

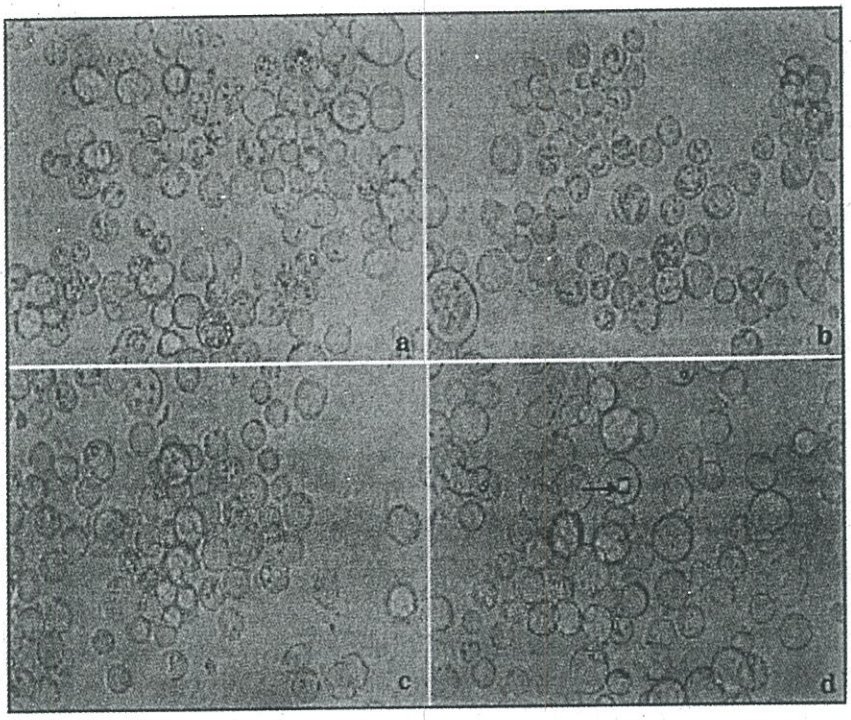

Figure 2 Phase-contrast micrographs of Sf9 cells infected with *Gm*NPV respectively from the 5th (a), 10th (b), 15th (c) and 20th (d) passages 48 hours post infection. The abnormal size of polyhedra (arrow) is shown in infected cell.

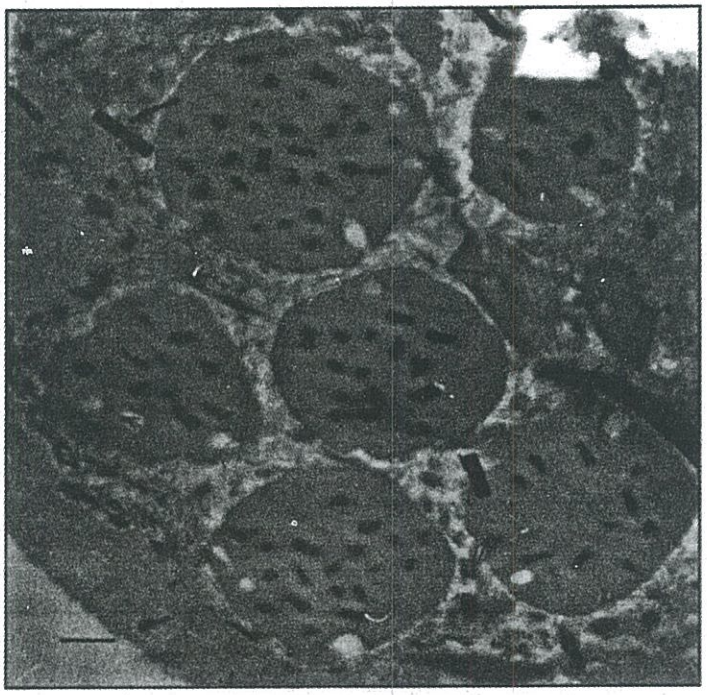

Figure 3 Electron micrograph of Sf9 cell infected with *Gm*NPV from the 1st passage. Bar = 500 nm

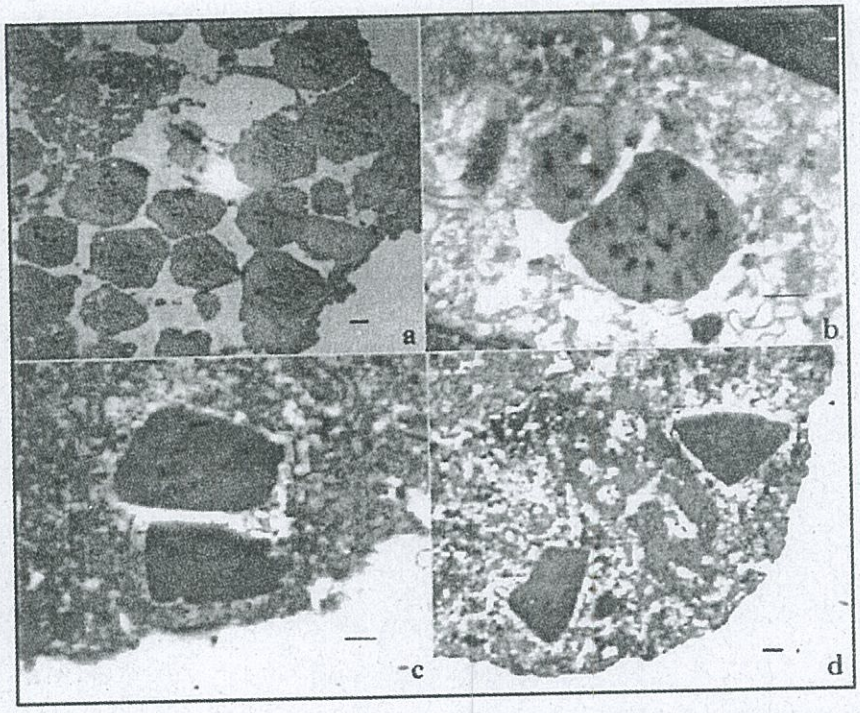

Figure 4 Electron micrographs of Sf9 cell infected with *Gm*NPV respectively from the 5th (a), 10th (b), 15th (c) and 20th (d) passages. Bar = 500 nm

polyhedra were observed which were abnormal both in shape and size. Inside the polyhedra there were very few or no occluded virions.

Microscopical observations were corroborated by quantitative evalution of polyhedra produced. As shown in Table 1, the polyhedra production declined slightly from the 1st passage up to 10th passage but after the 10th passage marked reduction in polyhedra number was observed. Statistical analysis of these data revealed that this reduction was significant ($P<0.05$).

The titer of the extracellular virus fluctuated from $1.58x10^7$ to $4.68x10^9$ $TCID_{50}$/ml, with the lowest titers detected in the 6th passage (Table 1). During the 9th and 10th passages the titers of extracellular virus were observed to increase about 1,000 and 1,000,000 folds when compared with the 1st passage. The data subjected to statistical analysis indicated that the ECV titers obtained from the 9th and 10th passages were significantly higher than from other passages ($P<0.05$).

The extracellular virus obtained from the 1st, 10th and 15th passages were plaque-purified 3 times in Sf9 cell line. In this experiment, five plaque-purified isolates were obtained (Table 2). In order to determine the production of polyhedra and titer of extracellular virus, each plaque-purified isolate was used as virus inoculum to infect Sf9 cells. The results indicated that one plaque purified isolate designated as P-10 exhibited high productivity of polyhedra and extracellular virus titer. In other words, this plaque-purified isolate exhibited either high infectivity or productivity. Therefore, the polyhedra produced from P-10 were further used in bioassay studies to compare their infectivity with the polyhedra produced from P-1, P-5, and wild type *Gm*NPV *to Plutella xylostella*.

Table 2 Plaque purified isolates

Plaque isolates	Total No. of polyhedra per culture (5 ml)	$TCID_{50}$/ml
P-1a	$3.55x10^5$	$6.31x10^8$
P-1b	$5.25x10^5$	$4.68x10^8$
P-10	$2.40x10^6$	$>10^{17}$
P-15a	$9.77x10^5$	$4.27x10^{16}$
P-15b	$1.02x10^6$	$5.62x10^{16}$

The comparative bioassays of the 1st, 5th, 10th passage isolates, plaque-purified isolate and wild isolate of *Gm*NPV to the 2nd stage larvae of diamondback moth were performed using leaf disk bioassay. Normally, signs of larval infection were observed after 2-3 days post infection. When the infected larvae were not fed, they turned to pale green color. Mortality of infected larvae was observed within 2 days after feeding was terminated. Dead larvae were dark brown in color and easily disrupted when gently touched by camel brush. The concentration-mortality responses and mean median lethal time values (LT_{50}s) data obtained are shown in Tables 3 and 4 respectively. The percentage mortality was calculated from the total number of dead infected larvae. The data were then submitted to Probit analysis [13]. According to the calculated median lethal concentration values (LC_{50}s), the wild isolate of *Gm*NPV was most pathogenic to second-instar of *Plutella xylostella* with the LC_{50} value of 1.94 $x10^4$ OBs/cm^2 (Table 3). It was found that the LC_{50} value of wild type *Gm*NPV was significantly higher than the 1st, 5th, 10th passage isolates and plaque purified isolate when treatment means were compared using ANOVA and LSD test ($P<0.05$). For the other 4 isolates, *Gm*NPV from 1st passage (LC_{50} = 5.68 $x10^4$ OBs/cm^2), 5th passage (LC_{50} = 6.75 $x10^4$ OBs/cm^2), 10th passage (LC_{50} = 6.89 $x10^4$ OBs/cm^2) and plaque-purified isolate (LC_{50} = 4.71 $x10^4$ OBs/cm^2) showed no significant different in virulence ($P>0.05$).

Table 3 Mean median lethal concentration values (LC_{50}s) of the 1st passage isolate, 5th passage isolate, 10th passage isolate, plaque-purified isolate and wild isolate of *Gm*NPV for 2nd stage larvae of *Plutella xylostella*

Virus isolate	Slope	LC_{50}(OBs/cm^2)	95% Fiducial limits
Wild type isolate	1.3190	1.94 $x10^{4\,a}$	1.37 $x10^4$-2.77 $x10^4$
1st passage isolate	1.2120	5.68 $x10^{4\,b}$	3.90 $x10^4$-8.79 $x10^4$
5th passage isolate	0.9785	6.75 $x10^{4\,b}$	4.28 $x10^4$-1.20 $x10^5$
10th passage isolate	1.1770	6.89 $x10^{4\,b}$	4.63 $x10^4$-1.11 $x10^5$
Plaque-purified isolate	1.2145	4.71 $x10^{4\,b}$	3.18 $x10^4$-7.40 $x10^4$

Means followed by the same letter were not significantly different ($P>0.05$, one-way ANOVA; LSD test)

Table 4 Mean median lethal time values (LT_{50}s) of the 1st passage isolate, 5th passage isolate, 10th passage isolate, plaque-purified isolate and wild isolate of *Gm*NPV for 2nd stage larvae of *Plutella xylostella*

Virus isolate	Slope	LT_{50} (days)	95% Fiducial limits
Wild type isolate	8.35	4.8 [a]	4.4-5.2
1st passage isolate	5.88	5.1 [a]	4.6-5.6
5th passage isolate	5.67	5.6 [a]	5.1-6.4
10th passage isolate	4.94	5.1 [a]	4.6-5.8
Plaque-purified isolate	6.83	5.4 [a]	4.9-5.9

Means followed by the same letter were not significantly different (P>0.05, one-way ANOVA; LSD test)

4. DISCUSSION

Serial passage of *Gm*NPV resulted in significant reductions in percentage of infection and polyhedra production, which were apparent after 10 passages in cell culture. Also, the titer of ECV production was increased significantly by the 9th and 10th passages. All of these observations were quite similar to the previous reports [16-22]. Namely, the reduction in polyhedra production decreased when passage level was increased. These results indicated that the normal virus could degenerate into defective forms, which produced low yield of polyhedra. The generation of these mutants during serial passage known as the serial passage effect when viruses are passaged several times *in vitro* by using ECV. These genetic changes result from alterations within the viral genome such as deletions and insertions of DNA in essential viral genes [23-26].

Effects of serial passages of *Gm*NPV in Sf9 cells adapted to serum free-medium were also recently investigated. Despite the fact that stability of recombinant protein expression over 18 serial passages was noted, serial passages of the *Gm*NPV *in vitro* resulted, as also demonstrated in our work, in abnormality of polyhedra morphology, and occlusion of virions as well as decrease of polyhedra production and cell infection rate in serum-free condition [27-28].

Serial passage of *Gm*NPV in cell culture or larvae by using ECV as virus inoculum always produces spontaneous mutants, which produce fewer polyhedra than the wild type virus [20]. These mutants termed as FP (few polyhedra) mutants are usually detected within three or four passages and become predominant in cell culture by the 6th passage. The electron microscopic observation indicated that most of the polyhedra produced by FP mutants were devoid of nucleocapsid, while others contained fewer nucleocapsids.

MacKinnon *et al.* [17] reported the presence of another class of mutants, termed defective interfering particles, which have been correlated with the interfering of normal virus replication and the reduction in virulence of the virus. Kool *et al.* [29] suggested that the presence of defective interference particles is perhaps associated with the formation of FP mutants in that the insertion sequences in FP mutants are located within the deletion sequences of defective interfering particles. It is well known that serial passage in cell culture frequently leads to generation of spontaneous mutations at high frequency. In this regard, changes in virulence of polyhedra for the target insect seem to be the major problem and have been identified in several types of viruses.

Fraser and Hink [20] used electron microscopy to investigate the reason for the reduction in virulence of polyhedra derived from FP mutants of *Gm*NPV. They found that FP infections proceed with a minimum amount of de novo membrane synthesis in the nucleus. Hence, most progeny nucleocapsids are released from the nucleus into the cytoplasm instead of being occluded within polyhedra. Primary membrane acquisition for the cell-released non-occluded virus of the FP mutants occurs by budding from the cell membrane. This would explain the smaller yield of occlusion bodies produced and the decrease in virulence of polyhedra to larvae from virus at the higher passage level as observed in these experiments. In contrast, MP (many polyhedra) infection

produces many nucleocapsids in the nucleus indicating that a high degree of de novo membrane synthesis takes place.

After the 10th passage the total number of polyhedra produced decreased significantly and several reports, exist on the low infectivity of polyhedra obtained from the late passage level [16-20, 22, 30]. Thus, only the polyhedra recovered from the 1st, 5th, 10th passages were used in bioassay study. The data obtained from comparative bioassay of five *Gm*NPV isolates (the 1st, 5th, and 10th passage isolates, plaque-purified isolate and wild isolate) indicated that *Plutella xylostella* larvae are susceptible to *Gm*NPV infection, showing the typical symptoms of NPV infection. Based on the LC_{50} values, the wild isolate of *Gm*NPV showed highest virulence to 2nd stage larvae of *Plutella xylostella* among 5 materials tested, with LC_{50} value of 1.94×10^4 OBs/cm^2. These differences were significantly different when statistically analyzed using ANOVA model ($P<0.05$). Among the three passaged isolates, the virulence of polyhedra derived from the 1st passage was found to be highest with the LC_{50} of 5.68×10^4 OBs/cm^2 while the 5th and 10th passaged isolates showed lower LC_{50} values not differing significantly from each other (6.75×10^4 OBs/cm^2 and 6.89×10^4 OBs/cm^2, respectively). However, among these passaged isolates the statistical analysis showed no significant differences in LC_{50} values. This suggests that accumulation of FP mutants was not notable during the 1st to 10th passages.

According to the plaque-purification study, there was one plaque-purified isolate designated as P-10 that exhibited high productivity of ECV and polyhedra. Because of the notable increase in ECV production this plaque-purified isolate was suspected to enhance the rate of systemic transmission within the insect. Consequently, this plaque-purified isolate was submitted to bioassay study in order to test the hypothesis. The data obtained from bioassay indicated that the virulence of P-10 isolate was higher than those of the passaged isolates, although, the differences among the plaque-purified isolate and the three passaged isolates were not statistically significant. From this result, it may be postulated that the plaque-purified isolate had undergone some genetic modifications that influenced the level of virus production. However, the mechanism involved in the generation of this plaque-purified isolate was not clearly understood.

The comparison of infectivity of different virus isolates for *Plutella xylostella* obtained from both current and previous studies are shown in Table 5.

Table 5 Infectivity of various virus isolates for *Plutella xylostella*

Virus isolate	Slope	LD_{50} (OBs/cm^2)	95% Fiducial limits
*Px*NPVCL3[a]	0.60	5.54	3.10-8.91
*Af*NPVCL1[a]	0.82	9.22×10^3	6.48×10^3-1.32×10^4
*Ac*NPV[a]	1.02	1.16×10^4	8.53×10^3-1.58×10^4
*Gm*NPV (wild isolate)[b]	1.32	1.94×10^4	1.37×10^4-2.77×10^4
1st passaged *Gm*NPV[b]	1.21	5.68×10^4	3.90×10^4-8.79×10^4
5th passaged *Gm*NPV[b]	0.98	6.75×10^4	4.28×10^4-1.20×10^5
10th passaged *Gm*NPV[b]	1.18	6.89×10^4	4.63×10^4-1.11×10^5
Plaque-purified isolate of *Gm*NPV[b]	1.12	4.71×10^4	3.18×10^4-7.40×10^4

[a] Infectivity bioassay was performed by using 1st stage larvae of *Plutella xylostella*
[b] Infectivity bioassay was performed by using 2nd stage larvae of *Plutella xylostella*

The concentration-mortality responses indicated that the plaque-purified isolate (PxNPVCL3) obtained from a new baculovirus isolate recovered from infected larvae of *Plutella xylostella* was most pathogenic to 1st stage larvae of *Plutella xylostella* (LC_{50} = 5.54 OBs/cm^2) followed by AfNPV (Anagrapha falcifera nuclear polyhedrosis virus) ($LC_{50} = 9.22 \times 10^3$ OBs/cm^2) and AcNPV ($LC_{50} = 1.16 \times 10^4$ OBs/cm^2 [31]. Based on the infectivity bioassay of Abdul Kadir [6] found that the pathogenicities of *Gm*NPV and AcNPV were similar. Therefore, it may be concluded that *Gm*NPV has moderate virulence in controlling *Plutella xylostella*, comparable with AfNPV and AcNPV; despite the developmental stage of the tested larvae being different the

comparison can be done. Because of the fact that the increasing age of tested larvae will affect the susceptibility of *Plutela xylostella* to NPVs, it is to be expected that the concentration needed to kill 50 percent of 2nd stage larvae of *Plutella xylostella* should be higher.

On the basis of time-mortality responses, the median lethal time values (LT_{50}s) of *Gm*NPV 5 isolates were calculated from the same concentration (10^7 OBs/ml). The time-mortality responses of *Gm*NPV 5 isolates at this dosage are shown in Table 4. All data were also subjected to Probit analysis and time-mortality response lines for 2nd stage larva of *Plutella xylostella* are shown in Table 4.

Although several NPVs have been reported to be pathogenic to *Plutella xylostella* including AcNPV, *Gm*NPV [5-6,31-33] AfNPV and PxNPVCL3 [34] up to now no one has used these viruses for controlling *Plutella xylostella* already stated. This current study is first report on the usage of *Gm*NPV and its plaque-purified isolate to control *Plutella xylostella* elsewhere. Large-scale production of *Gm*NPV based biopesticide media should be developed to obtain easily prepared and low-cost formulation for use on crops. In addition, long-term passage of *Gm*NPV by using ECV is not recommended because the infectivity and polyhedra production of this virus began to decline after 10 passages. Usage of alkaline solution or alkaline solution and insect gut fluid could diminish such problems but it still led to the reduction in polyhedra production when passage level was increased [35]. However, the reduction in polyhedra production was observed in lesser degree than that observed in passage with ECV. Fraser and Hink [20] suggested that serial passage of *Gm*NPV in insect host by using PDV could also avoid serial passage effect.

Furthermore, in attempt to use *Gm*NPV as biocontrol agent several trials should be carried out including formulation, host range determination and field application study. In Thailand NPVs are accepted and NPV of *Spodoptera exigua* and *Heliothis armigera* have already been used in integrated pest management programs for a long time. These viruses have been developed and produced by Department of Agriculture in Bangkok using laboratory-reared larvae.

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